

THE EFFECT OF LITHIUM AND O-IODOSOBENZOIC ACID ON THE EARLY DEVELOPMENT OF THE SEA URCHIN EGG

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In 1892 C. Herbst observed that the addition of lithium ions to sea water changes the determination of the sea urchin egg in a vegetal direction, *i.e.*, the development of the endoderm becomes accentuated, whereas the differentiation of the ectoderm is suppressed, leading to a vegetalization of the larva. This specific effect of lithium has been utilized in a number of important investigations on developmental physiology; for references, see, for example, Gustafson (1954), Hörstadius (1935, 1939, 1949), Lindahl (1936, 1941), Runnström (1954), von Ubusch (1953).

The vegetalizing action of lithium becomes apparent in an exogastrulation of the larva, *i.e.*, there is no invagination of the gut; the ciliation of the larva is also reduced. These aberrant morphological characteristics are not manifested, however, until a rather late stage of development, whereas the treatment, which gives rise to the changes referred to above, may be applied in immediate connection with fertilization. The direct effect of lithium on the larva during the actual period of treatment does not seem, as yet, to have been studied in detail.

Animalization (the opposite of vegetalization), which is manifested as an increase in the development of the ectoderm and a reduced differentiation of the endoderm, has likewise been achieved by various treatments (*cf.* Lindahl, 1941; Runnström, 1954). These so-called animalized larvae show a strong ciliation and a suppressed development of the gut. It has recently been demonstrated that o-iodosobenzoic acid (IBA) exerts a strong animalizing action on sea urchin larvae (Bäckström, 1953; Runnström and Kriszat, 1952a, 1952b); the treatment with IBA is also applied during the early development of the larva, during the first hours after fertilization.

In 1952, the author observed that lithium interferes with cell division, and also the movements of the chromosomes were found to be affected when lithium ions in concentrations of 0.05–0.06 *M* were present in the sea water. Owing to other investigations in progress, these first observations on the cytological effect of lithium were not followed up until 1959, when the present work was started.

MATERIAL AND METHODS

The present study was carried out at Stazione Zoologica in Naples, where *Paracentrotus lividus* and *Psammechinus microtuberculatus* served as material. Experiments have also been made at Biologisk Stasjon, Espesgrend, Norway, with eggs and sperm from *Echinus esculentus* and *Strongylocentrotus droebachiensis*, and at Kristinebergs Zoologiska Station, Fiskebäckskil, Sweden, where the gametes from *Echinocardium cordatum* and *Psammechinus miliaris* were used.

In the experiments with lithium the concentrations varied from 0.03 *M* to 0.09 *M*. The concentrations of *o*-iodosobenzoic acid used varied between 5×10^{-5} *M* and 10^{-3} *M*.

The experimental treatment with lithium and IBA was applied according to several different methods. In most experiments the active substances were added to batches of the egg suspension within 5–10 minutes after insemination. At different time intervals after the first treatment was started, new batches of eggs from the fertilized control were transferred to the lithium and IBA. In other series, lithium and IBA were added to the larvae when they were passing through a certain cell phase, *e.g.* prophase or metaphase, or when they were just completing cell division, *e.g.* from the 4- to the 8-cell stage.

In most of the experiments the treatment with the active substances was interrupted at the 64–128-cell stage, and the larvae were transferred to pure sea water; in other series, the larvae were reared in the presence of lithium or IBA through the hatching stage.

The cultures with cleaving larvae were counted at different time intervals after insemination; the percentages of cleavage given in this paper are based on counts of 200–250 larvae. The counts were made either in the living state or after fixation in Carnoy's fluid or in 4% formol. When the counts were made on the living cultures, the control and the cultures treated were in most experiments inseminated successively at intervals of 5 minutes, which enables the counts to be made at the same point of time after insemination.

Studies of the chromosomes and the nuclear phases were carried out after fixation of the larvae in Carnoy's fluid and staining with aceto-carmin or aceto-orcein.

RESULTS

The effect of lithium on the rate of cleavage

When fertilized eggs were transferred to sea water containing lithium, a considerable retardation in the rate of cleavage was observed. In experiments where the inseminated eggs were subjected to lithium already before the sperm and egg nuclei had fused, this retardation became particularly evident, often resulting in a complete blocking of the first cell division. This was especially noticeable when the concentration of lithium was kept rather high, *i.e.*, 0.06–0.09 *M*.

A certain variation in sensitivity to lithium between the different species tested was observed and, for example, in *Strongylocentrotus droebachiensis* and *Echinus esculentus*, species which develop at a rather low temperature, and consequently the first cleavage requires three hours or more to be completed, the blocking effect on the fusion of the pronuclei was more pronounced than in *Paracentrotus*. The latter species develops more rapidly and at a much higher temperature. The retardation noted at the first cleavage is evident also during the ensuing development.

In other experiments the treatment with lithium was started at different time intervals after the first cell division, and also in these experiments the retarding effect of lithium on cleavage was striking. The sensitivity to lithium was tested in consecutive experiments up to the 64- to 128-cell stage, when it becomes difficult to observe the single cell divisions.

The effect of o-iodosobenzoic acid on the rate of cleavage

Experiments similar to those described with lithium were carried out also with o-iodosobenzoic acid. Most of these experiments were made at the same time as those with lithium; the same egg material and the same control were used for both series.

In contrast to lithium, IBA was found to effect a general increase in the rate of cleavage. As in the experiments with lithium, treatment with IBA was started before the first cell division, and at different intervals during cleavage, *i.e.*, at the 2-, 4-, 8-, 16-, 32-, and 64-cell stages. The length of treatment was also varied as in the lithium experiments. Thus, the treatment was stopped, for instance, after the 16-cell stage or at the 64- to 128-cell stage. Experimental series were also carried out in which the treatment was not interrupted until after the larvae in the control had begun to hatch.

More than 200 experiments, in which the larvae were reared over hatching, were made in the course of the present investigation. In many of these experiments the development was followed up to the pluteus stage. Though the experiments were carried out with six species of sea urchins, under varied and different experimental conditions, the results were practically uniform. The results of a representative experiment with eggs and sperm from *Paracentrotus lividus* are referred to in Table I.

TABLE I

Eggs and sperm from Paracentrotus lividus. Temperature 18.5° C. (1) Control. The eggs were inseminated with 10⁶ spermatozoa per ml. Twenty minutes after insemination eggs from the control were transferred to: (2) 0.06 M LiCl, and (3) 3.5 × 10⁻⁴ M IBA

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|---------------------------------|---|
| 45 minutes after insemination: | No cleavage was observed in either (1), (2) or (3) |
| 58 minutes after insemination: | (1) 90% uncleaved, 10% 2-cell (2) 100% uncleaved (3) 40% uncleaved, 60% 2-cell |
| 76 minutes after insemination: | (1) 3% uncleaved, 97% 2-cell (2) 7% uncleaved, 93% 2-cell (3) 97% 2-cell, 3% 4-cell |
| 90 minutes after insemination: | (1) 99% 2-cell, 1% 4-cell (2) 3% uncleaved, 97% 2-cell (3) 85% 2-cell, 15% 4-cell |
| 135 minutes after insemination: | (1) 2% 2-cell, 50% 4-cell, 48% 8-cell (2) 3% 2-cell, 85% 4-cell, 12% 8-cell (3) 8% 4-cell, 92% 8-cell |
| 190 minutes after insemination: | (1) 4% 8-cell, 96% 16-cell (2) 53% 8-cell, 47% 16-cell (3) 89% 16-cell, 11% 32-cell |

The absolute difference in time between the control and the batches of eggs developing in the presence of lithium or IBA is, among other factors, dependent upon the species used, the concentration of the active substance and the temperature. The retardation obtained in the presence of 0.06 *M* lithium was measured to about 70 minutes for the second cleavage of eggs from *Strongylocentrotus droebachiensis* reared at 8° C. The corresponding retardation observed for eggs from *Paracentrotus* at 19.5° C. was recorded to about 12–15 minutes. At 18° C. this difference may amount to 20 minutes or more. The advance in cleavage obtained by treating the larvae in 7×10^{-4} *M* IBA was found to be 20–25 minutes for the second to fourth cleavages of *Paracentrotus* eggs reared at a temperature of 18.4° C.

As has already been pointed out, many factors influence the rate of cleavage. Experiments of the type shown in Table I have been preferred for evaluating the qualitative effect of the substances tested, whereas in experiments where the absolute retarding or enhancing effect was to be studied, the cultures were fixed at the same stage of development and the difference in time was measured.

The influence of lithium and IBA on the fertilization rate

A sensitive method of testing the positive or negative effect of substances present in fertilization experiments is the so-called fertilization rate method (Hagström and Hagström, 1954, 1959).

Because both lithium and IBA were found to exert a strong influence on early development, it was thought to be of interest to investigate the action of these substances also on fertilization.

Lithium and IBA were added to the egg suspensions immediately before insemination, and consequently, the substances acted on both eggs and spermatozoa at the moment of fertilization. After 5, 10, 15, 20, etc., seconds, sodium lauryl sulphate was added to a final concentration of 0.001%, which instantaneously kills the spermatozoa without affecting the eggs (Hagström and Hagström, 1954, 1959). This method makes it possible to count the number of eggs fertilized 5, 10, 15, etc., seconds after insemination.

The concentrations of lithium in the present experiments were 0.04 *M* and 0.06 *M*, and the concentrations of IBA varied between 3×10^{-4} *M* and 10^{-3} *M*.

Lithium in the concentrations tested produced no effect on the fertilization rate. In the experiments with IBA there was also no clear difference recorded when compared with the control series. In some cases, however, there was a slight improvement with IBA in concentrations of about 10^{-3} *M*. No deleterious effects on the processes at fertilization due to either lithium or IBA were observed. This is of importance for the evaluation of the cleavage rate experiments.

The influence of lithium and IBA on hatching

The first developmental period ends when, at hatching, the sea urchin larva breaks the fertilization membrane and can swim freely. The series of events leading up to hatching makes it possible to record a number of objective observations on the rate of development.

As previously mentioned, the duration of treatment varied, and in some experiments it was interrupted at a relatively early stage, *i.e.*, in the 64- to 128-cell stage,

whereas in other experiments the active substances were present from a few minutes after insemination until the larvae started to hatch in the control. Many variations were applied within this general scheme of treatment.

In experiments where the larvae were reared in lithium or IBA from insemination until the larvae of the control began to hatch, these substances were found definitely to inhibit hatching. It is known that IBA prevents hatching, and that the larvae may then develop inside the fertilization membrane (Runnström and Kriszat, 1952b). The present experiments showed that lithium also tended to arrest hatching. There is, however, a considerable difference between the action of lithium and that of IBA. Lithium not only delays or completely inhibits hatching, but also prevents the formation of cilia and the movements of the larva inside the intact membrane. In IBA the actual rupture of the membrane is obviously impeded, whereas the ciliation of the larva is normally or even more than normally developed. Consequently, in the presence of IBA the larvae acquire a high degree of rotatory activity inside the membranes. This is also in agreement with the fact that the larvae develop more rapidly in IBA; accordingly, the viability of the larvae seems to be high. A typical experiment is referred to in Table II.

In certain experiments with high concentrations of lithium and IBA, hatching was completely arrested, especially when the slowly developing Norwegian species were used.

Experiments in which the active substances were removed at a relatively early stage of development were also carried out. Qualitatively, the same results were gained as in the experiments mentioned above; lithium gives rise to a general decrease in the rate of development, whereas IBA promotes development, with the exception that IBA affects hatching.

The negative effect exerted by IBA on hatching must be ascribed to the fact that the rupture of the fertilization membrane is arrested, and that this process is not correlated with any retardation of the development in general.

Morphological observations on the cleavage stages

The effect of lithium and IBA on the nuclei and chromosomes appeared to be of special importance. Observations were made with the phase contrast microscope on vital material, and, moreover, about 1500 fixations have been prepared during the course of this investigation. The results of the cytological studies will be reported elsewhere. The present paper will deal only with observations on the cleavage pattern of the young larva.

With IBA, cleavage seems to be normal though accelerated. The cleavage furrows are deep, but the blastomeres remain well attached to each other.

The effects observed after treatment with lithium were considerably more complex. Lithium was found to induce a very clear separation of the blastomeres. This deterioration in the contacts between the cells of the cleaving blastulae is likely to affect the transport mechanism within the larva and the exchange of metabolites between the different regions of the embryo, thus interfering with a mechanism which is undoubtedly of the utmost importance to the ensuing development. When the micromeres form, they seem, however, to retain intercellular contact with each other and with the macromeres. This may be due to the fact that the micromeres have a small volume but a relatively large surface area as compared with the macro-

TABLE II

Paracentrotus lividus, eggs from one female. Temperature 18° C. (1) Control. The eggs were inseminated with 10^6 spermatozoa per ml. Twelve minutes after insemination eggs from the control were transferred to: (2) 0.06 M LiCl, and
(3) 7×10^{-4} M IBA

9 hours 50 minutes after insemination:

- (1) A few ciliated larvae rotating inside the membranes. No hatched larvae.
- (2) No ciliation or hatching.
- (3) Most larvae ciliated and moving inside the membranes. No hatched larvae.

10 hours 15 minutes after insemination:

- (1) Increased ciliation and movements. No hatching.
- (2) No ciliation.
- (3) Vigorous movements, stronger than in (1). No hatching.

10 hours 40 minutes after insemination:

- (1) A few hatched larvae.
- (2) No ciliation, no movements or hatching.
- (3) Strong rotation inside the membranes. No hatching.

11 hours 10 minutes after insemination:

- (1) About 50% hatched larvae.
- (2) A few larvae with weak ciliation.
- (3) Vigorous movements, a few hatched larvae.

11 hours 55 minutes after insemination:

- (1) About 80% hatched larvae.
- (2) About 25% with weak rotating movements. No hatching.
- (3) Vigorous movements, about 40% hatched larvae.

12 hours 20 minutes after insemination:

- (1) 95% hatched larvae.
- (2) No hatching.
- (3) About 60% hatched larvae.

12 hours 55 minutes after insemination:

- (1) 100% hatched larvae.
- (2) No hatching, weak rotation inside the membranes.
- (3) About 80% ruptured membranes.

14 hours after insemination:

- (2) About 30% ruptured membranes. The ciliation was still very weak.
- (3) 90% hatched larvae. The larvae showed higher mobility than in the control.

14 hours 55 minutes after insemination:

- (2) 80% with ruptured membranes. Low mobility.
- (3) 100% hatched larvae.

16 hours 20 minutes after insemination:

- (2) 90% hatched larvae swimming near the bottom with low mobility.
- (3) The primary mesenchyme was well developed in (3) but was not present in the control or in (2).

meres. The position of the micromeres, squeezed in between the macromeres, may also facilitate intercellular contacts between these two types of cells.

As pointed out previously, lithium interferes with the rate of cleavage, and

TABLE III

Paracentrotus lividus, eggs from one female. Temperature 18° C. (1) Control. The eggs were inseminated with 10⁶ spermatozoa per ml. Eggs from the control were transferred into 0.05 M LiCl after: (2) 5 minutes; (3) 215 minutes

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| 120 minutes after insemination: | (1) 39% uncleaved, 61% 2-cell (2) 94% uncleaved, 6% 2-cell |
| 140 minutes after insemination: | (1) 1% uncleaved, 99% 2-cell (2) 52% uncleaved, 48% 2-cell |
| 165 minutes after insemination: | (1) 78% 2-cell, 22% 4-cell (2) 5% uncleaved, 95% 2-cell |
| 200 minutes after insemination: | (1) 27% 2-cell, 70% 4-cell, 3% 8-cell (2) 87% 2-cell, 13% 4-cell |
| 230 minutes after insemination: | (1) 64% 4-cell, 33% 8-cell, 3% 16-cell (2) 48% 2-cell, 48% 4-cell, 4% 8-cell (3) 65% 4-cell, 33% 8-cell, 2% 16-cell |
| 260 minutes after insemination: | (1) 14% 4-cell, 68% 8-cell, 18% 16-cell (2) 15% 2-cell, 74% 4-cell, 11% 8-cell (3) 3% 2-cell, 20% 4-cell, 66% 8-cell, 11% 16-cell |
| 290 minutes after insemination: | (1) 56% 8-cell, 44% 16-cell (2) 18% 2-cell, 42% 4-cell, 33% 8-cell, 7% <i>12-cell</i> (3) 3% 4-cell, 56% 8-cell, 2% <i>12-cell</i> , 39% 16-cell |
| 320 minutes after insemination: | (1) 26% 8-cell, 71% 16-cell, 3% <i>32-cell</i> (2) 15% 2-cell, 29% 4-cell, 10% 8-cell, 40% <i>12-cell</i> , 6% 16-cell (3) 2% 4-cell, 40% 8-cell, 5% <i>12-cell</i> , 53% 16-cell |
| 365 minutes after insemination: | (1) 6% 8-cell, 62% 16-cell, 32% <i>32-cell</i> (2) 2% 2-cell, 2% 4-cell, 6% 8-cell, 28% <i>12-cell</i> , 62% 16-cell (3) 7% 8-cell, 17% <i>12-cell</i> , 62% 16-cell, 14% <i>32-cell</i> |

within the larvae there appears to be a certain gradation in response to lithium; the different morphological regions of the embryo seem to be differently affected.

When the larvae have reached the 8-cell stage, and the process of cell division begins, which leads to the 16-cell stage, some of the embryos formed intermediate 12-cell stages (italicized in Table III) instead of the normal 16-cell stages. The cleavages in the four animal cells of the larva became temporarily inhibited when influenced by lithium, whereas the cleavages of the vegetal cells were not arrested to the same extent. The blocking of the cleavages in the animal region of the larvae is incomplete and temporary. As a consequence of the delayed formation of the presumptive mesomeres, the balance between the animal and the vegetal halves of the embryo becomes disturbed. The delayed cleavages in the animal half are also

evident at the formation of the 32-, 64- and 128-cell stages, when larvae with reduced numbers of animal cells occur frequently. A typical experiment is shown in Table III.

DISCUSSION

The results obtained in the present investigation indicate that the effects, which are recorded on cleavage after treatment with IBA and lithium, are correlated with the "animalization" and "vegetalization" observed during the ensuing development of the larvae. The enhanced or delayed rate of cleavage is not necessarily the direct cause of the animalizing or vegetalizing effect, but, in the author's opinion, the alteration in the rate of cleavage reflects the primary changes in the cells of the young larva, which result in the secondary events that occur during the later development.

The present observations indicate that the nuclei and chromosomes are affected by lithium, which interferes also with cell division. It has been shown that lithium ions cause a deficiency in the nucleoprotein synthesis in *Xenopus* embryos (Thomason, 1957), and this finding has probably some bearing on the present results.

The disruptive action of lithium on the cell contacts appears to be of special importance, because this is likely to render difficult the exchange of metabolites within the embryo. A phenomenon, which cannot be fully explained at present, is the high proportion of 12-cell stages (instead of 16-cell stages) observed after treatment with lithium. The fact that the reduced number of cells is dependent upon temporarily inhibited cleavages in the presumptive mesomeres indicates that the equilibrium within the larva is disturbed, and that the vegetal part of the embryo obtains a certain lead in development over the animal part. It may also indicate that the animal cells are more sensitive than the vegetal to exposure to lithium. The cell divisions are not equal, however (*cf.* Hörstadius, 1935), in the four animal and the four vegetal cells of the 8-cell stage, and consequently, they are not directly comparable. This indicates that the apparent differences in sensitivity to lithium may as readily be ascribed to the different orientation of the mitotic spindles in the animal and the vegetal cells, which may *per se* dispose the cells to respond differently to lithium. As has been previously mentioned, a similar effect of lithium was observed when the larvae were treated at the 16- to 32-cell stage, at the 32- to 64-cell stage, and at the 64- to 128-cell stage; larvae with less than the normal number of cells were also frequently encountered here.

Whether this disturbance of the cleavage pattern is to be attributed to a real difference in sensitivity between the animal and the vegetal cells of the embryo may, at present, be left an open question. At the cleavage stages between 16 and 128 cells, the cells of the animal and the vegetal halves are rather unequal. These cells have not the same surface areas, and the ratio, surface area/volume, is also different in the animal half from that in the vegetal. If we assume that the uptake and the action of lithium are correlated with the surface area exposed to the active ions, it may be justifiable to conclude that the deleterious effect on the animal part of the larva is visible on account of the larger surface area exposed to lithium. However, if there is any graded response to lithium in the different parts of the embryo, it is likely to be of a temporary nature. The elaborate experiments carried out by Lindahl and Holter (1940) point in the same direction. Their results

showed that isolated animal and vegetal halves have the same oxygen consumption. Furthermore, it was demonstrated that treatment with lithium evokes the same inhibition of respiration in both animal and vegetal cells (Lindahl and Holter, 1940).

It is evident that lithium causes a general slowing down of early development. Lindahl (1936, 1941) showed that the oxygen consumption of larvae reared in the presence of lithium is considerably lower than that for larvae which develop in pure sea water; this is in agreement with the results reported here. The lithium effect becomes especially marked at the time of hatching, when the ciliation of the larvae treated is either reduced or entirely lacking. In some experiments, hatching took place before any ciliation of the larvae had developed, which may indicate that the production of the hatching enzyme (*cf.* Lundblad, 1954) is less affected by lithium than are the processes leading to ciliation.

The results obtained with IBA show that the action of this substance enhances cleavage. The ciliation of the larva is extremely well developed, and the rotatory movements inside the membrane often begin earlier in a treated batch of larvae than in the corresponding control. The actual rupture of the membrane is, however, delayed or even prevented. It was previously shown (Bäckström, 1955) that IBA does not interfere with the respiration of the larva up to the hatching stage, which is in accordance with the present results. IBA has not, as yet, been observed to cause any unbalanced increase in the cleavage of, for example, the animal half of the larva, but all cells within the embryo seem to be subject to the same effect that promotes cleavage. Though a number of substances and treatments have been found to induce animalization of the sea urchin larva, no common denominator for their physiological action has so far been discovered. It is therefore of interest to note that trypsin, which also induces an animalizing effect (Hörstadius, 1949, 1953), has recently been shown to bring about an accelerated rate of cleavage comparable with that resulting from treatment with IBA (Hagström and Löming, 1962).

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SUMMARY

Under varied experimental conditions young sea urchin larvae were subjected to the action of solutions of o-iodosobenzoic acid and LiCl in sea water. Cleavage and early development were found to be advanced by IBA, and retarded by lithium. The fusion of the pronuclei was strongly inhibited by lithium, which tends also to separate the blastomeres. The rate of fertilization was not appreciably influenced by either IBA or lithium in the concentrations tested.

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